Effect of food-related stress conditions and loss of agr and sigB on seb promoter activity in S. aureus

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DOI: https://doi.org/10.1016/j.fm.2017.03.006

Manuscript Details

Manuscript number	FM_2016_979
Title	Effect of food-related stress conditions and loss of agr and sigB on seb promoter activity in S. aureus
Article type	Research Paper

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Keywords	staphylococcal enterotoxin B; stress response; agr; sigB

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Zurich, Dec 1st, 2016

Dear Editor,

Please find enclosed the manuscript “Effect of food-related stress conditions and loss of *agr* and *sigB* on promoter activity in *S. aureus*” for publication in Food Microbiology.

Staphylococcal enterotoxin B (SEB) causes foodborne intoxications and is produced in up to ten times higher quantities than other major enterotoxins. While *Staphylococcus aureus* growth is often repressed by competing flora, the organism exhibits a decisive growth advantage under some stress conditions. The aim of this study was to investigate the influence of stress and regulatory mutations on *seb* promoter activity.

We were able to show that NaCl, nitrite, and glucose stress led to significantly decreased *seb* promoter activity, while lactic acid stress resulted in significantly increased *seb* promoter activity. Loss of *agr* decreased *seb* promoter activity and loss of *sigB* increased promoter activity. These results demonstrate that mild stress conditions encountered during food production and preservation can induce significant changes in *seb* promoter activity.

I declare that the manuscript has not been submitted or accepted for publication elsewhere. Moreover, I warrant that all authors have seen and approved the manuscript and have contributed significantly to this work.

Thank you and best wishes from Switzerland.

Sincerely,

Dr. Sophia Johler

Effect of food-related stress conditions and loss of *agr* and *sigB* on *seb* promoter activity in *S. aureus*

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Running Head: The effect of stress and regulatory mutations on *seb* promoter activity

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ABSTRACT

Staphylococcal enterotoxin B (SEB) causes staphylococcal food poisoning and is produced in up to ten times higher quantities than other major enterotoxins. While *Staphylococcus aureus* growth is often repressed by competing flora, the organism exhibits a decisive growth advantage under some stress conditions. So far, data on the influence of food-related stressors and regulatory mutations on *seb* expression is limited and largely based on laboratory strains, which were later reported to harbor mutations. Therefore, the aim of this study was to investigate the influence of stress and regulatory mutations on *seb* promoter activity. To this end, transcriptional fusions were created in two strains, USA300 and HG003, carrying different *seb* upstream sequences fused to a *blaZ* reporter. NaCl, nitrite, and glucose stress led to significantly decreased *seb* promoter activity, while lactic acid stress resulted in significantly increased *seb* promoter activity. Loss of *agr* decreased *seb* promoter activity and loss of *sigB* increased promoter activity, with the magnitude of change depending on the strain. These results demonstrate that mild stress conditions encountered during food production and preservation can induce significant changes in *seb* promoter activity.

Keywords: *Staphylococcus aureus,* staphylococcal enterotoxin B*,* stress response*, agr, sigB*

1. Introduction

Staphylococcal food poisoning is one of the most common foodborne intoxications worldwide. Enterotoxins are produced by enterotoxigenic *Staphylococcus (S.) aureus* strains. Enterotoxin B (SEB) is one of the major enterotoxins responsible for food poisoning outbreaks and is produced in ten times higher quantities compared to other enterotoxins such as SEA, SED, and SEE (Bergdoll 1979). Expression of prophage encoded SEA and SEE appears to be highest at early-exponential growth phase, while SEB, SEC, and SED production is temporally regulated and higher quantities are produced post-exponentially (Derzelle et al., 2009; Lis et al., 2012; Schelin et al., 2011). Highest SEB concentrations are produced during the transition from the exponential to the stationary phase of growth (Bergdoll, 1979; Czop & Bergdoll, 1974; Otero, Garcia, Garcia, Moreno, & Bergdoll, 1990). This expression pattern coincides with the activity of Agr, a *S. aureus* quorum sensing system that causes a post-exponential increase in the transcription of exotoxins (Dunman et al., 2001; Bronner et al., 2004,). The effector of the Agr system, the regulatory RNA RNAIII, influences exotoxin expression both directly by binding to mRNAs and indirectly by inhibiting other gene regulators such as the repressor of toxins, Rot (Morfeldt et al., 1995; Tseng and Stewart, 2005). A role for Agr in the regulation of *seb*, *sec*, and *sed* expression has been proposed (Bronner et al., 2004; Thoendel et al., 2011; Bronesky et al., 2016).

However, most studies investigating the effect of regulatory mutations on enterotoxin expression have been conducted using derivatives of strain NCTC8325 harboring an 11-base deletion in *rsbU*, a gene encoding an indirect positive regulator of SigB (Gertz et al., 1999). Since a defect in the *sigB* operon has been shown to affect global regulators Agr, Sar, and Rot, results generated using NCTC8325 derivatives may not be representative for other commonly found *S. aureus* strains (Cassat et al., 2006; Lauderdale et al., 2009). In addition, many previous studies

neglected the important factor of strain-specific variation (Begley and Hill, 2015; Rode et al., 2010; Troller, 1971) and relied exclusively on immunological methods, although it has been shown that loss of serological activity does not equal loss of emetic activity (Bennett and Berry, 1987).

S. aureus is highly resistant to stress conditions encountered in various food matrices and is able to outcompete competitive flora under low a_w (water activity) or acidic conditions. Various environmental stress conditions post-translationally activate the alternative sigma factor SigB, which functions antagonistically to Agr (Novick, 2003). SigB also increases the expression of staphylococcal accessory regulator (*sarA*) (Bischoff et al., 2001). While there is data available on the production of several enterotoxins under optimal growth conditions (pH 7, no NaCl or nitrite stress), data on enterotoxin production under stress conditions is lacking (Derzelle et al., 2009; Lee et al., 2007).

In this study, the influence of four food-related stress conditions (NaCl, nitrite, glucose, lactic acid stress) as well as regulatory mutations (Δ*agr*, Δ*sigB*) on *seb* promoter activity was investigated using transcriptional fusions of different *seb* upstream regions and a *blaZ* reporter in two different reference strain backgrounds.

2. Materials and methods

2.1. Bacterial strains

S. aureus strains and plasmids used in this study are listed in Table 1. USA300 is a community-aquired methicillin resistent *S. aureus* (Diep et al., 2006) and HG003 is a NCTC8325 derivative with restored *rsbU* and *tcaR* genes (Herbert et al., 2010). Isogenic regulatory knockout mutants (Δ*agr*, Δ*sigB*) were constructed by transduction using phage 80α as previously described (Charpentier et al., 2004; Sihto et al., 2015).

2.2. Construction of SEBp blaZTT transcriptional fusions

A 1 kb region upstream of the *seb* gene of the three different SEB producing strains COL, S6C, and KLT6 (further referred to as C, S, and K), was amplified by PCR. Each of these *seb* upstream regions was cloned into a pGEM-T Easy cloning vector (Promega, Fitchburg, WI) and the vectors were transformed into *E. coli* Top10. The inserts were cloned into pCN41 (Charpentier et al., 2004) using BamHI and PstI sites, and the *ermC* cassette was exchanged for the *cat194* cassette (Charpentier et al., 2004), before transformation of the constructs into Top10. The constructs were subsequently moved to pJC1648 and pJC1649, shuttle vectors, for which the SaPI1/ SaPI3 att_s cassette had been replaced by a SaPI4 att_s cassette. The constructs were electroporated into a derivate of RN4220 expressing the SaPI4 integrase and were transduced to the USA300 and HG003 target strains using phage 80α.

2.3. Growth conditions

Single colonies were transferred from 5% sheep blood agar to 5 mL of LB broth (Bertani 1959) supplemented with cadmium or erythromycin and grown for 18 h (37°C, 200 rpm). Primary day cultures in LB (10 mL) were adjusted to OD_{590} = 0.05, grown until mid-exponential phase (ca. 2 h), and washed to remove residual media components and to avoid carryover of AIP (centrifugation at 8000 *x* g for 3 min). Cells were resuspended in 1 mL of LB only (control) or LB adjusted to the respective stress condition (4.5% NaCl, 150 mg/L nitrite, 30% glucose, lactic acid pH 6.0, respectively). Stress levels were chosen to reflect stress conditions encountered in food products. Secondary day cultures were adjusted to the desired OD (OD_{590} = 0.03 - 0.10 depending on growth condition) and incubated at 37°C, 200 rpm. Samples were harvested 2 h, 4 h, 6 h, and 24 h after inoculation, corresponding to mid-exponential (2h), late exponential (4h), early stationary (6h), and stationary phase (24h). Two independent cultivations were performed for all strains to gain two independent samples of each strain, condition, and time point.

2.4. β-lactamase assay

The activity of the reporter gene *blaZ* was determined using a nitrocefin assay as previously described (O'Callaghan et al., 1972; Yoon et al., 1991). Samples were diluted 1:10 on the 96-well plate and 50 µL of nitrocefin (Merck, Darmstadt, Germany) was added. The nitrocefin concentration equalled 132 µg/mL in 100 nM sodium phosphate buffer (pH 5.8). Colorimetric cleavage of nitrocefin was monitored by measuring OD_{490} in a microplate reader spectrophotometer (BioTek, Winooski, VT). Maximum enzymatic activity was determined from kinetic data using Gen5 (BioTek, Winooski, VT) and defining the initial slope (Vmax). Vmax values were normalized using OD 600 nm to minimize variation caused by differences in cell densities.

2.5. Statistical analysis

Statistically significant differences in *seb* promoter activity under stress compared to control conditions as well as in regulatory mutant strains compared to isogenic wild types (wt) were defined using Student's t-test. Statistical analysis was performed using SPSS Statistics 23 (SPSS Inc., Chicago, IL). Results were considered significant at $P < 0.05$.

3. Results

3.1. Impact of strain background and seb upstream region on seb promoter activity

OD_{590} measurements (Supplementary Fig. 1) demonstrated a similar increase in cell density between strains under control and stress conditions, with the exception of glucose stress affecting growth. The activity of three different 1 kb fragments upstream of *seb* (K, C, S) fused to a *blaZ* reporter gene was investigated in a USA300 and a HG003 background. Generally, *seb* promoter activity was higher in the USA300 background compared to the HG003 background. No

statistically significant differences related to the three 1kb *seb* upstream regions (K, C, S) were detected.

3.2. Impact of stress on seb promoter activity

Under NaCl stress, *seb* promoter activity was significantly reduced compared to the control conditons in LB in USA300 background strains (time points 2 h, 4 h, 6 h, 24 h) as well as in HG003 background strains (time points 4 h, 6 h, 24 h) (Fig. 1A). Under nitrite stress, *seb* promoter activity was significantly reduced in most USA300 background strains at earlier time points (4 h, 6 h) and in most HG003 background strains at later time points (6 h, 24 h) (Fig. 1B). Glucose stress had the most prominent effect on *seb* promoter activity and in particular in HG003, the *seb* promoter seemed generally unresponsive under glucose stress. In all tested strains, *seb* promoter activity was significantly reduced at 6 h and 24 h (72% - 90% decrease depending on the strain) (Fig. 1C). Comparison of *seb* promoter acitivity under lactic acid stress (pH 6.0) was compared to pH-stabilized control samples in LB, at pH 7.0. Interestingly, under lactic acid stress, a trend towards higher *seb* promoter activity was observed (Fig. 1D). Both under control and stress conditions, *seb* promoter activity increased with increasing cell density and *seb* promoter activity was highest at late stationary phase (at 24 h). See Supplementary Table 1 for *p*-values related to each condition and time point.

3.3. Impact of regulatory mutations on seb promoter activity

Regulatory mutants lacking *agr* exhibited significantly lower *seb* promoter activity in all strains ($p < 0.05$ at all time points except at 2 h) (Fig. 2A). The change was most pronounced at 24 h, with a 52% - 63% decrease depending on the strain. Maximum *seb* promoter activity in Δ*agr* mutants was reached at late stationary phase (24 h), similar to control and stress conditions. In contrast, in most *sigB* mutants, maximum *seb* promoter activity was reached in the early stationary phase (Fig. 2B). In addition, regulatory mutants lacking *sigB* exhibited significantly

higher *seb* promoter activity in most USA300 and HG003 background strains compared to the wt. A marked strain-specific difference was observed in the impact of *sigB* on *seb* promoter activity. In the USA300 background, a maximum increase of 78% was observed, as opposed to a maximum increase of 1027% in the HG003 background.

4. Discussion

All tested food-related stress conditions resulted in significant changes in *seb* promoter activity, but with temporal and strain-specific differences. The changes in *seb* promoter activity under stress conditions are consistent with studies investigating enterotoxin D expression under stress conditions (Sihto et al., 2015; Sihto et al., 2016a;. Sihto et al., 2016b). Glucose and NaCl stress conditions resulted in reduced *seb* promoter activities, in line with earlier studies investigating enterotoxin protein levels by immunological methods (Ewald and Notermans, 1988; Genigeorgis et al., 1971; Genigeorgis and Sadler, 1966; Iandolo and Shafer, 1977; Jarvis et al., 1975). While glucose stress suppressed *seb* promoter activity at all tested time points, maximum suppression by NaCl stress was observed at 4h and 6h.

Nitrite stress caused less pronounced effect on *seb* promoter activity. On protein level, it has been shown that *S. aureus* growth and SEB production remain unaffected at nitrite concentrations of up to 200 mg/L (McLean et al., 1968). Interestingly, lactic acid stress led to slightly increased *seb* promoter activity. Similar findings were recently reported for enterotoxin A, suggesting induced enterotoxin A expression under acidic stress conditions (Rosengren et al., 2013; Wallin-Carlquist et al., 2010; Zeaki et al., 2015). On the protein level, enterotoxin production has been shown to be highest between pH 6–7 (Genigeorgis et al., 1971; Genigeorgis and Sadler, 1966).

Our results also show that the strain background had an effect on the level of *seb* promoter activity. While we generally observed far lower *seb* promoter activities in a HG003 than in a

USA300 background, loss of *sigB* led to an extreme increase in *seb* promoter activity in HG003 background strains. These results suggest that repression of *seb* promoter activity observed in a HG003 background is largely due to SigB activity. It could be hypothesized that acquisition of mutations affecting the functionality of SigB and thus increasing the synthesis of toxins may offer a competitive advantage in a clinical context. This may also have been the case for NCTC8325 harboring an 11-base deletion in *rsbU*, a gene encoding an indirect positive regulator of SigB (Gertz et al., 1999).

Compared to other *S. aureus* lineages, USA300 strains have been shown to exhibit increased expression of *agr* and *saeRS,* which could partly explain higher *seb* promoter activity levels compared to HG003 (Montgomery et al., 2008).

Overall, no significant effect of different *seb* upstream sequences on *seb* promoter activity was observed. However, at 4h, HG003_S harboring the *seb* upstream element of S6C exhibited consistently lower *seb* promoter activities than HG003_K and HG003_C, harboring *seb* upstream sequences of KLT6 and COL, respectively. These findings support data by Sato'o et al. (Sato'o et al., 2013) suggesting that SEB production may depend on the SaPI carrying *seb* rather than the *seb* promoter region.

With regard to the regulatory mutants in our study, strain-specific differences were evident. Loss of *agr* resulted in higher reduction in *seb* promoter activity in USA300 derivatives compared to HG003 and loss of *sigB* generated higher induction in *seb* promoter activity in HG003 derivatives compared to USA300. These and similar findings highlight the importance of including several *S. aureus* strains in studies investigating regulatory elements (Schmidt et al., 2004; Sihto et al., 2016b). Interestingly, *seb* promoter activity in *sigB* mutants peaked at 6 h, while highest activity under all other conditions was observed at 24 h. In contrast, in *agr* mutants,

highest *seb* promoter activity levels were already reached at 6 h, with similarily high levels after 24 h in all strains apart from HG003_S.

In conclusion, mild stress conditions encountered during food production and preservation can induce significant changes in *seb* promoter activity. In addition, *agr* and *sigB* play a major role in *seb* regulation.

Acknowledgments

This work was supported by grants from the Swiss National Research Program 69 (40690_145211/1); a Forschungskredit of the University of Zurich (grant no. FK-13-059 and FK15-055); and a grant from the Hermann Herzer Foundation. Funding sources were not involved in the study design, data collection, or analysis.

The authors would like to express their gratitude to Richard P. Novick for helpful discussions and for providing laboratory space and strains, and to Brigitte Berger-Bächi for providing strains.

References

Begley, M., Hill, C., 2015. Stress adaptation in foodborne pathogens. Annu. Rev. Food Sci. Technol 6, 191–210. http://dx.doi.org/10.1146/annurev-food-030713-092350

Bennett, R.W., Berry, M., 1987. Serological reactivity and in vivo toxicity *Staphylococcus aureus* enterotoxins A and D in selected canned foods. J. Food Sci. 52, 416–418.

Bergdoll M. S., 1979. Staphylococcal intoxications, in: Riemann H., Bryan F. L. (Eds.), Foodborne infections and intoxications. Academic Press, New York, pp 443–493.

Bischoff, M., Entenza, J.M., Giachino, P., 2001. Influence of a functional *sigB* operon on the global regulators *sar* and *agr* in *Staphylococcus aureus*. J. Bacteriol. 183, 5171–5179. http://dx.doi.org/10.1128/JB.183.17.5171

Bronesky, D., Wu, Z., Marzi, S., Walter, P., Geissmann, T., Moreau, K., Caldelari, I., Romby, P., 2016. *Staphylococcus aureus* RNAIII and its regulon link quorum sensing, stress responses,

metabolic adaptation, and regulation of virulence gene expression. Ann. Rev. Microbiol. 70, 299–316. http://dx.doi.org/10.1146/annurev-micro-102215-095708

Bronner, S., Monteil, H., Prévost, G., 2004. Regulation of virulence determinants in *Staphylococcus aureus*: complexity and applications. FEMS Microbiol. Rev. 28, 183–200. http://dx.doi.org/10.1016/j.femsre.2003.09.003

Cassat, J., Dunman, P.M., Murphy, E., Projan, S.J., Beenken, K.E., Palm, K.J., Yang, S.-J., Rice, K.C., Bayles, K.W., Smeltzer, M.S., 2006. Transcriptional profiling of a *Staphylococcus aureus* clinical isolate and its isogenic *agr* and *sarA* mutants reveals global differences in comparison to the laboratory strain RN6390. Microbiology 152, 3075–3090. http://dx.doi.org/10.1099/mic.0.29033-0

Charpentier, E., Anton, A.I., Barry, P., Alfonso, B., Fang, Y., Novick, R.P., 2004. Novel cassette-based shuttle vector system for Gram-positive bacteria. Appl. Environ. Microbiol. 70, 6076–6085. http://dx.doi.org/10.1128/AEM.70.10.6076

Czop, J.K., Bergdoll, M.S., 1974. Staphylococcal enterotoxin synthesis during the exponential, transitional, and stationary growth phases. Infect. Immun. 9, 229–235.

Derzelle, S., Dilasser, F., Duquenne, M., Deperrois, V., 2009. Differential temporal expression of the staphylococcal enterotoxins genes during cell growth. Food Microbiol. 26, 896–904. http://dx.doi.org/10.1016/j.fm.2009.06.007

Diep, B.A., Gill, S.R., Chang, R.F., Phan, T.H., Chen, J.H., Davidson, M.G., Lin, F., Lin, J., Carleton, H.A., Mongodin, E.F., Sensabaugh, G.F., Perdreau-Remington, F., 2006. Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant *Staphylococcus aureus*. Lancet 367, 731–739. http://dx.doi.org/10.1016/S0140-6736(06)68231-7

Dunman, P.M., Murphy, E., Haney, S., Palacios, D., Tucker-Kellogg, G., Wu, S., Brown, E.L., Zagursky, R.J., Shlaes, D., Projan, S.J. 2001. Transcription profiling-based identification of *Staphylococcus aureus* genes regulated by the *agr* and/or *sarA* loci. J. Bacteriol. 183:7341–7345

Ewald, S., Notermans, S., 1988. Effect of water activity on growth and enterotoxin D production of *Staphylococcus aureus*. Int. J. Food Microbiol. 6, 25–30.

Genigeorgis, C., Foda, M.S., Mantis, A., Sadler, W.W., 1971. Effect of sodium chloride and pH on enterotoxin C production. Appl. Microbiol. 21, 862–866.

Genigeorgis, C., Sadler, W.W., 1966. Effect of sodium chloride and pH on enterotoxin B production. J. Bacteriol. 92, 1383–1387.

Gertz, S., Engelmann, S., Schmid, R., Ohlsen, K., Hacker, J., Hecker, M., 1999. Regulation of sigmaB-dependent transcription of *sigB* and *asp23* in two different *Staphylococcus aureus*

strains. Mol. Gen. Genet. 261, 558–66.

Gill, S.R., Fouts, D.E., Archer, G.L., Mongodin, E.F., Deboy, R.T., Ravel, J., Paulsen, I.T., Kolonay, J.F., Brinkac, L., Beanan, M., Dodson, R.J., Daugherty, S.C., Madupu, R., Angiuoli, S. V, Durkin, a S., Haft, D.H., Vamathevan, J., Khouri, H., Utterback, T., Lee, C., Dimitrov, G., Jiang, L., Qin, H., Weidman, J., Tran, K., Kang, K., Hance, I.R., Nelson, K.E., Fraser, C.M., 2005. insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain. J. Bacteriol. 187, 2426–2438. doi:10.1128/JB.187.7.2426

Herbert, S., Ziebandt, A.-K., Ohlsen, K., Schäfer, T., Hecker, M., Albrecht, D., Novick, R., Götz, F., 2010. Repair of global regulators in *Staphylococcus aureus* 8325 and comparative analysis with other clinical isolates. Infect. Immun. 78, 2877–2889. http://dx.doi.org/10.1128/IAI.00088-10

Iandolo, J.J., Shafer, W.M., 1977. Regulation of staphylococcal enterotoxin B. Infect. Immun. 16, 610–616.

Jarvis, A.W., Lawrence, R.C., Pritchard, G.G., 1975. Glucose repression of enterotoxins A, B and C and other extracellular proteins in staphylococci in batch and continuous culture. J. Gen. Microbiol. 86, 75–87.

Lauderdale, K.J., Boles, B.R., Cheung, A.L., Horswill, A.R., 2009. Interconnections between Sigma B, *agr*, and proteolytic activity in *Staphylococcus aureus* biofilm maturation. Infect. Immun. 77, 1623–1635. http://dx.doi.org/10.1128/IAI.01036-08

Lee, Y.-D., Moon, B.-Y., Park, J.-H., Chang, H.-I., Kim, W.J., 2007. Expression of enterotoxin genes in *Staphylococcus aureus* isolates based on mRNA analysis. J. Microbiol. Biotechnol. 17, 461–467.

Lis, E., Podkowik, M., Bystroń, J., Stefaniak, T., Bania, J., 2012. Temporal expression of staphylococcal enterotoxin H in comparison with accessory gene regulator–dependent and –independent enterotoxins. J. Food Prot. 75, 238–244. http://dx.doi.org/10.4315/0362-028X.JFP-11-336

McLean, R.A., Lilly, H.D., Alford, J.A., 1968. Effects of meat-curing salts and temperature on production of staphylococcal enterotoxin B. J. Bacteriol. 95, 1207–1211.

Montgomery, C.P., Boyle-Vavra, S., Adem, P. V, Lee, J.C., Husain, A.N., Clasen, J., Daum, R.S., 2008. Comparison of virulence in community-associated methicillin-resistant *Staphylococcus aureus* pulsotypes USA300 and USA400 in a rat model of pneumonia. J Infect. Dis. 198, 561–570. http://dx.doi.org/10.1086/590157

Morfeldt, E., Taylor, D., von Gabain, A., Arvidson, S., 1995. Activation of alpha-toxin translation in *Staphylococcus aureus* by the trans-encoded antisense RNA, RNAIII. EMBO

J. 14, 4569–4577.

Novick, R.P., 2003. Autoinduction and signal transduction in the regulation of staphylococcal virulence. Mol. Microbiol. 48, 1429–1449. http://dx.doi.org/10.1046/j.1365-2958.2003.03526.x

O'Callaghan, C.H.O., Morris, A., Kirby, S.M., Shingler, A.H., 1972. Novel method for detection of *β*-lactamases by using a chromogenic cephalosporin substrate. Antimicrob. Agents Chemother. 1, 283–288.

Otero, A., Garcia, M.L., Garcia, M.C., Moreno, B., Bergdoll, M.S., 1990. Production of staphylococcal enterotoxins C1 and C2 and thermonuclease throughout the growth cycle. Appl. Environ. Microbiol. 56, 555–559.

Rode, T.M., Møretrø, T., Langsrud, S., Langsrud, O., Vogt, G., Holck, A., 2010. Responses of *Staphylococcus aureus* exposed to HCl and organic acid stress. Can. J. Microbiol. 56, 777–792. http://dx.doi.org/10.1139/W10-057

Rosengren, A., Lindblad, M., Lindqvist, R., 2013. The effect of undissociated lactic acid on *Staphylococcus aureus* growth and enterotoxin A production. Int. J. Food Microbiol. 162, 159–166. http://dx.doi.org/10.1016/j.ijfoodmicro.2013.01.006

Sato'o, Y., Omoe, K., Ono, H.K., Nakane, A., Hu, D.-L., 2013. A novel comprehensive analysis method for *Staphylococcus aureus* pathogenicity islands. Microbiol. Immunol. 57, 91–99. http://dx.doi.org/10.1111/1348-0421.12007

Schelin, J., Cohn, M.T., Barker, G.C., 2011. The Formation of *Staphylococcus aureus* enterotoxin in food environments and advances in risk assessment. Virulence 2, 580–592.

Schmidt, K.A., Donegan, N.P., Kwan, W.A., Cheung, A., 2004. Influences of *sigma B* and *agr* on expression of staphylococcal enterotoxin B (*seb*) in *Staphylococcus aureus*. Can. J. Microbiol. 50, 351–360. http://dx.doi.org/10.1139/W04-017

Sihto, H.-M., Tasara, T., Stephan, R., Johler, S., 2015. Temporal expression of the staphylococcal enterotoxin D gene under NaCl stress conditions. FEMS Microbiol. Lett. 362, 1–7. http://dx.doi.org/10.1093/femsle/fnv024

Sihto, H.-M., Tasara, T., Stephan, R., Johler, S., 2016a. Growth behavior and temporal enterotoxin D expression of *Staphylococcus aureus* strains under glucose and lactic acid stress. Food Control 62, 69–73. http://dx.doi.org/10.1016/j.foodcont.2015.10.008

Sihto, H.M., Budi Susilo, Y., Tasara, T., Rådström, P., Stephan, R., Schelin, J., Johler, S., 2016b. Effect of sodium nitrite and regulatory mutations Δ*agr*, Δ*sarA*, and Δ*sigB* on the mRNA and protein levels of staphylococcal enterotoxin D. Food Control 65, 37–45. http://dx.doi.org/10.1016/j.foodcont.2016.01.007

Thoendel, M., Kavanaugh, J.S., Flack, C.E., Horswill, A.R., 2011. Peptide signaling in the

Staphylococci. Chem. Rev. 111, 117–151. http://dx.doi.org/10.1021/cr100370n

Tseng, C.W., Stewart, G.C., 2005. Rot repression of enterotoxin B expression in *Staphylococcus aureus*. J. Bacteriol. 187, 5301–5309. http://dx.doi.org/10.1128/JB.187.15.5301

Tobes, R., Manrique, M., Brozynska, M., Stephan, R., Pareja, E., Johler, S., 2013. Noncontiguous finished genome sequence of *Staphylococcus aureus* KLT6, a staphylococcal enterotoxin B-positive strain involved in a food poisoning outbreak in Switzerland. Genome Announc. 1, 49239. http://dx.doi.org/10.1128/genomeA.00214-13

Troller, J.A., 1971. Effect of water activity on enterotoxin B production and growth of *Staphylococcus aureus*. Appl. Microbiol. 21, 435–439.

Wallin-Carlquist, N., Cao, R., Márta, D., da Silva, A.S., Schelin, J., Rådström, P., 2010. Acetic acid increases the phage-encoded enterotoxin A expression in *Staphylococcus aureus*. BMC Microbiol. 10, 1–10. http://dx.doi.org/10.1186/1471-2180-10-147

Yoon, K.P., Misra, T.K., Silver, S., 1991. Regulation of the *cadA* cadmium resistance determinant of *Staphylococcus aureus* plasmid pI258. J. Bacteriol. 173, 7643–7649.

Zeaki, N., Rådström, P., Schelin, J., 2015. Evaluation of potential effects of NaCl and sorbic acid on staphylococcal enterotoxin A formation. Microorganisms 3, 551–566. http://dx.doi.org/10.3390/microorganisms3030551

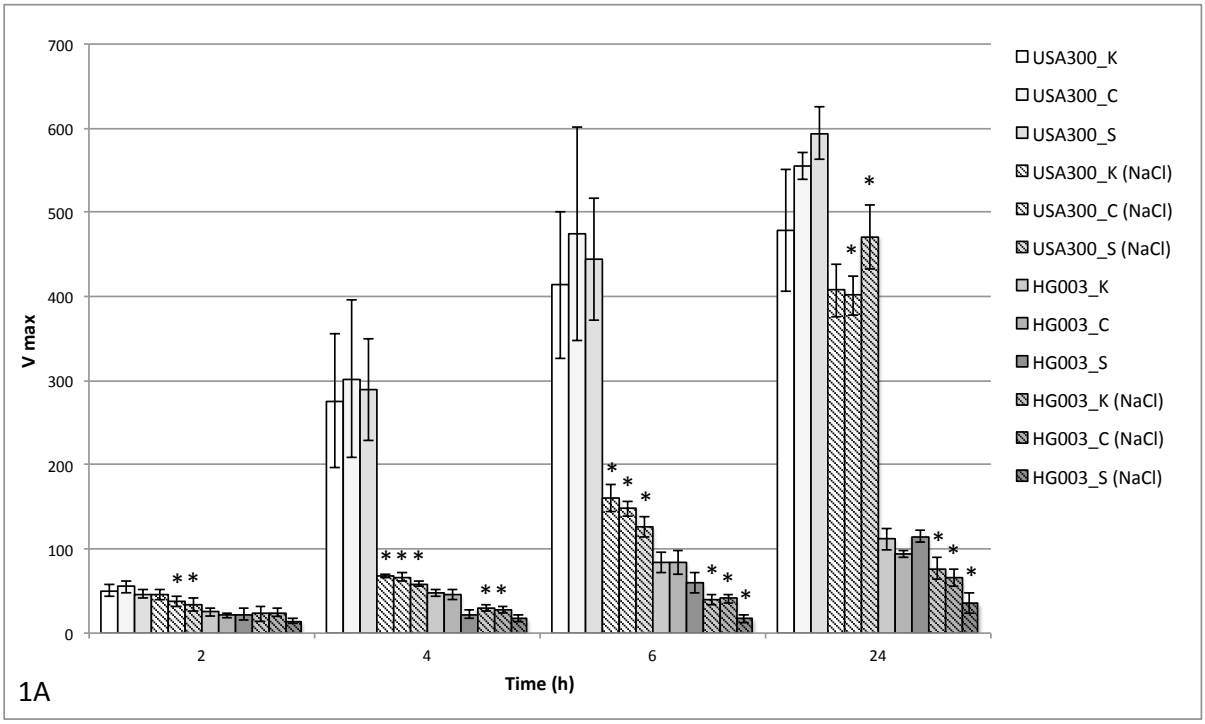
Figure legends

Fig. 1.

Normalized Vmax values stating *seb* promoter activity of USA300 and HG003 strain derivates under control (LB) and stress conditions A) 4.5 % NaCl; B) 150 mg/L nitrite; C) 30% glucose; D) lactic acid (pH 6.0). Error bars represent one standard deviation of the mean of two independent experiments performed in triplicates. Statistically significant changes between control and stress conditions at the same time point in each strain are marked by an asterisk ($P < 0.05$).

Fig. 2.

Normalized Vmax values stating *seb* promoter activity of USA300 and HG003 strain derivates and their isogenic regulatory mutants. A) Wild type and Δ*agr* mutant strains in LB; B) Wild type and Δ*sigB* mutant strains in LB. Error bars represent one standard deviation of the mean of two independent experiments performed in triplicates. Statistically significant changes between wild type and regulatory mutants at the same time point are marked by an asterisk ($P < 0.05$).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400
300
200
100
0
V max
2
4
6
24
Time (h)
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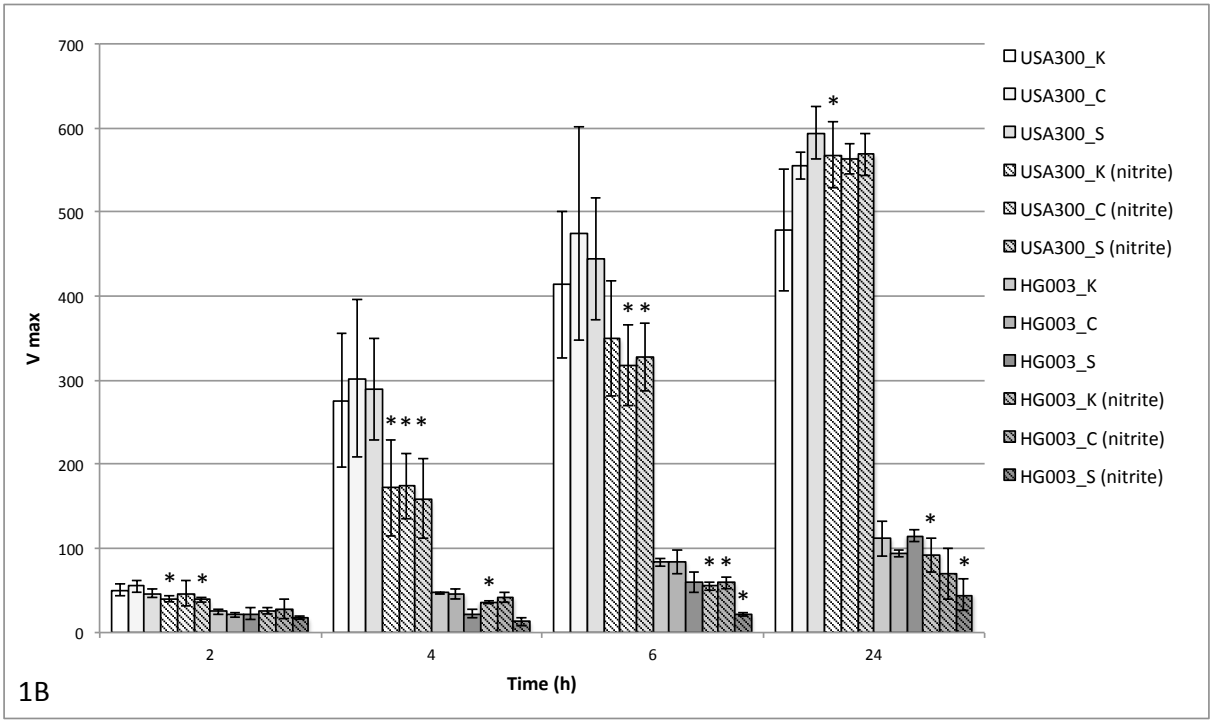
USA300_C
USA300_S
USA300_K (NaCl)
USA300_C (NaCl)
USA300_S (NaCl)
HG003_K
HG003_C
HG003_S
HG003_K (NaCl)
HG003_C (NaCl)
HG003_S (NaCl)

1A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600
500
400
300
200
100
0
V max
2
4
6
24
Time (h)
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USA300_C
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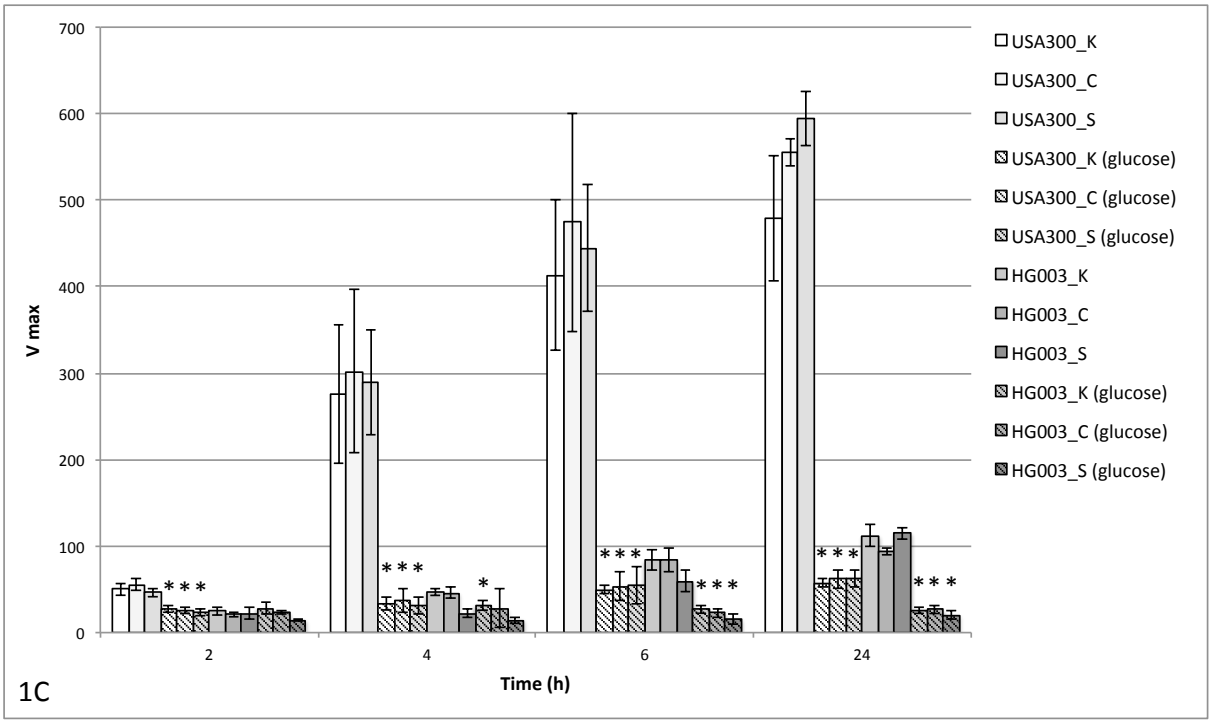
USA300_K (nitrite)
USA300_C (nitrite)
USA300_S (nitrite)
HG003_K
HG003_C
HG003_S
HG003_K (nitrite)
HG003_C (nitrite)
HG003_S (nitrite)

1B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600
500
400
300
200
100
0
V max
2
4
6
24
Time (h)
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USA300_C
USA300_S
USA300_K (glucose)
USA300_C (glucose)
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HG003_C
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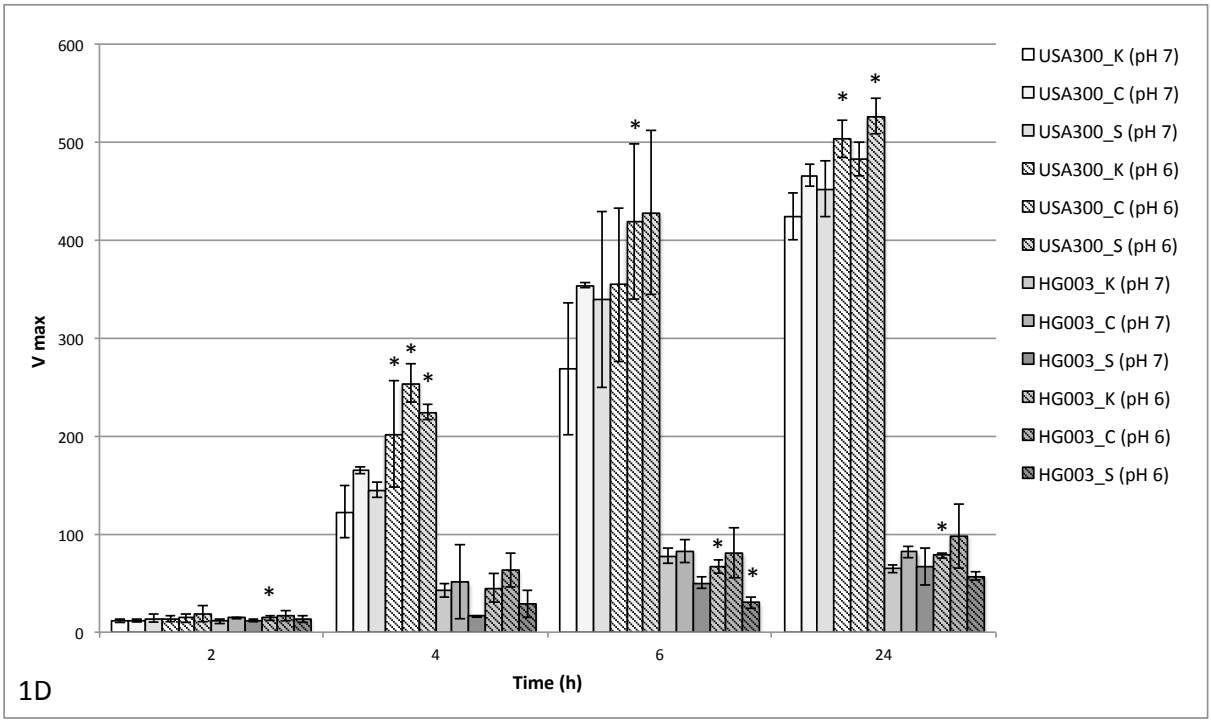
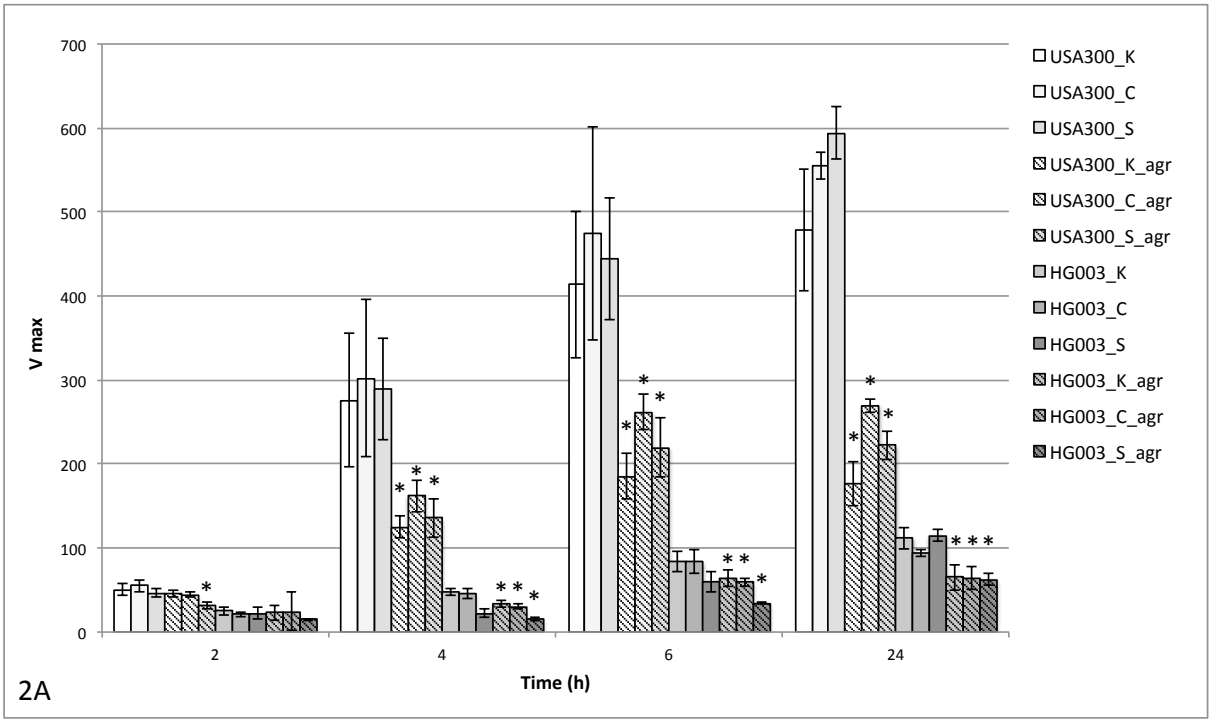
HG003_K (glucose)
HG003_C (glucose)
HG003_S (glucose)

1C


600
500
400
300
200
100
0
V max
2
4
6
24
Time (h)
USA300_K (pH 7)
USA300_C (pH 7)
USA300_S (pH 7)
USA300_K (pH 6)
USA300_C (pH 6)
USA300_S (pH 6)
HG003_K (pH 7)
HG003_C (pH 7)
HG003_S (pH 7)
HG003_K (pH 6)
HG003_C (pH 6)
HG003_S (pH 6)

1D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500
400
300
200
100
0
V max
2
4
6
24
Time (h)
USA300_K
USA300_C
USA300_S
USA300_K_agr
USA300_C_agr
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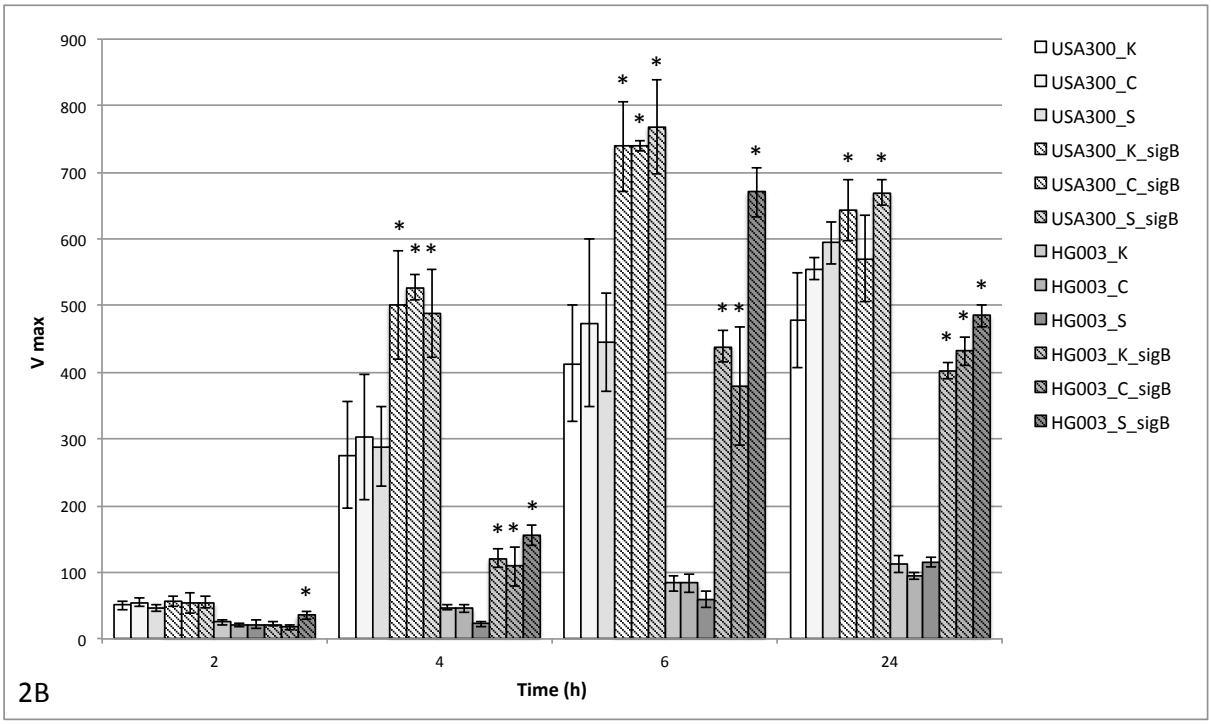
HG003_C_agr
HG003_S_agr

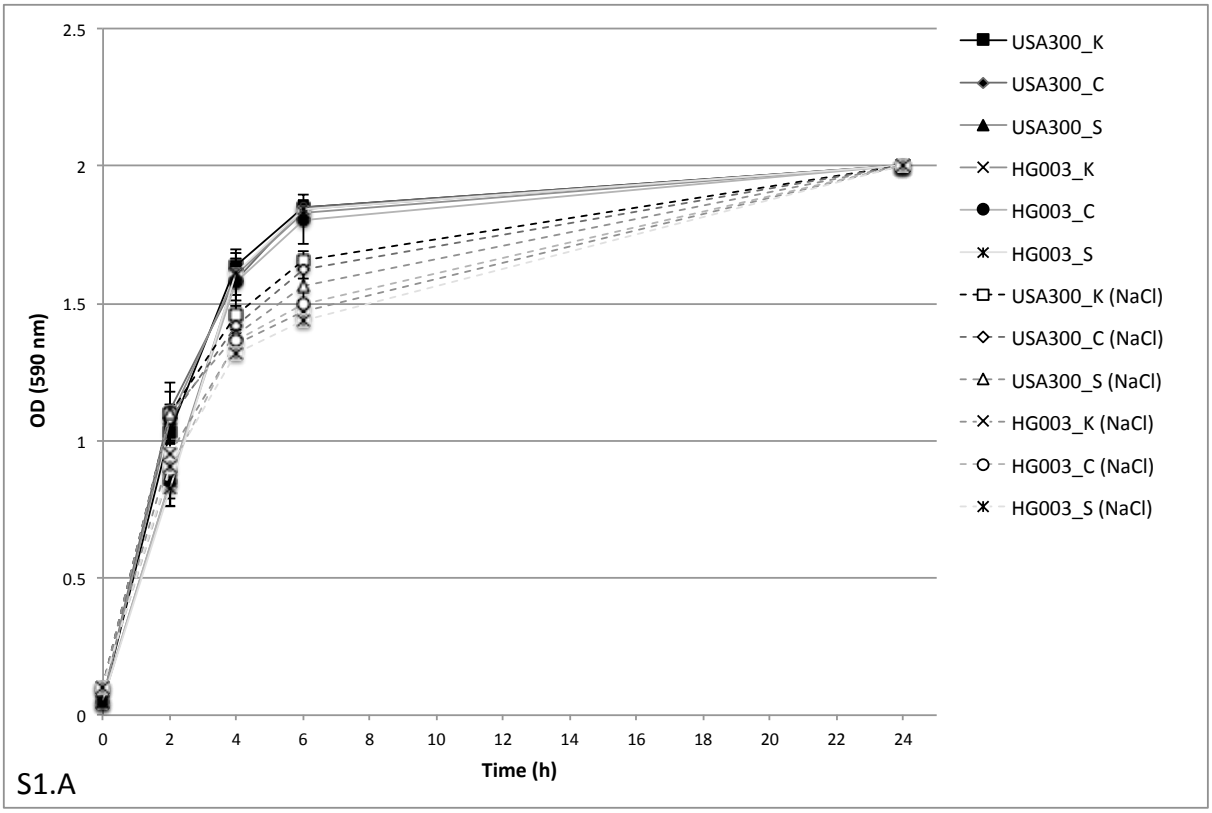
2A


900
800
700
600
500
400
300
200
100
0
V max
2
4
6
24
Time (h)
USA300_K
USA300_C
USA300_S
USA300_K_sigB
USA300_C_sigB
USA300_S_sigB
HG003_K
HG003_C
HG003_S
HG003_K_sigB
HG003_C_sigB
HG003_S_sigB

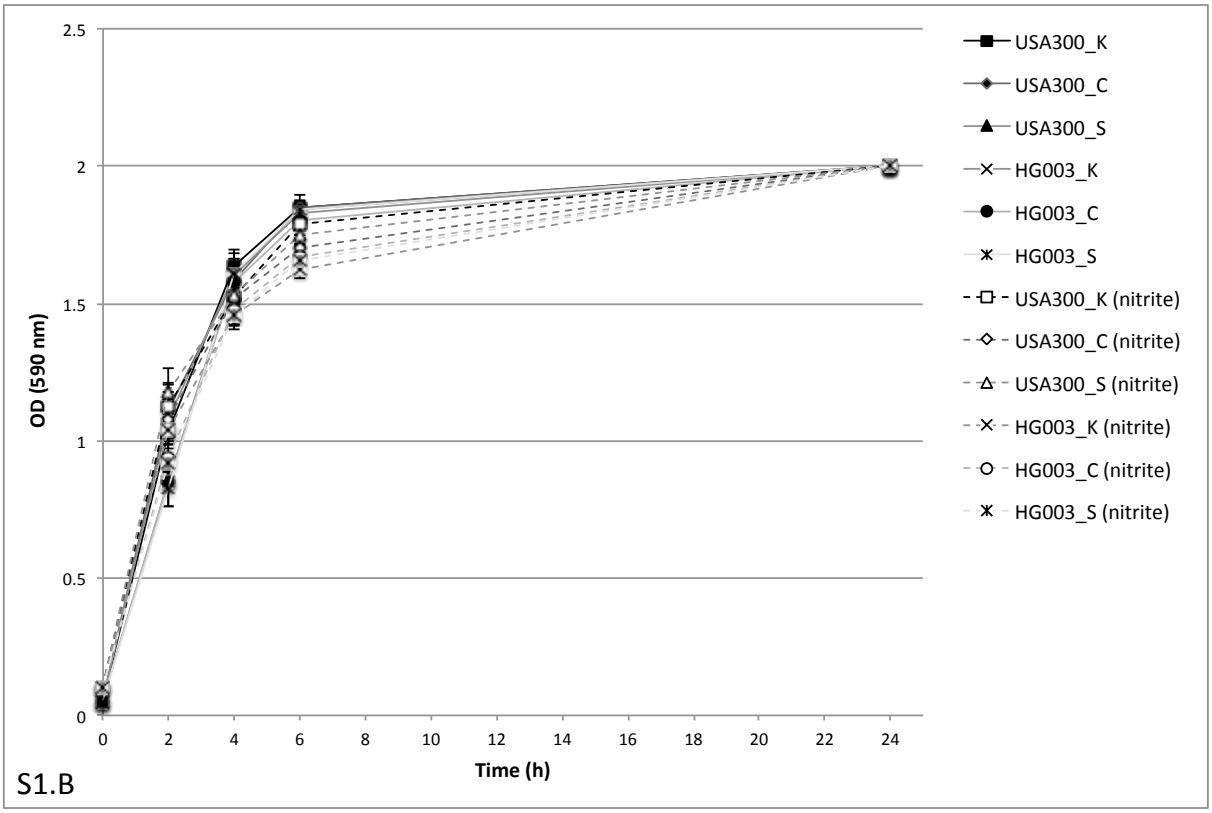
2B

Supplementary Fig. 1.

Increase in cell density expressed as OD_{590} values under control (LB) and stress conditions. A) 4.5 % NaCl; B) 150 mg/L nitrite; C) 30% glucose; D) lactic acid (pH 6.0). Error bars represent one standard deviation of the mean of two independent experiments.


OD (590 nm)
Time (h)
USA300_K
USA300_C
USA300_S
HG003_K
HG003_C
HG003_S
USA300_K (NaCl)
USA300_C (NaCl)
USA300_S (NaCl)
HG003_K (NaCl)
HG003_C (NaCl)
HG003_S (NaCl)

S1.A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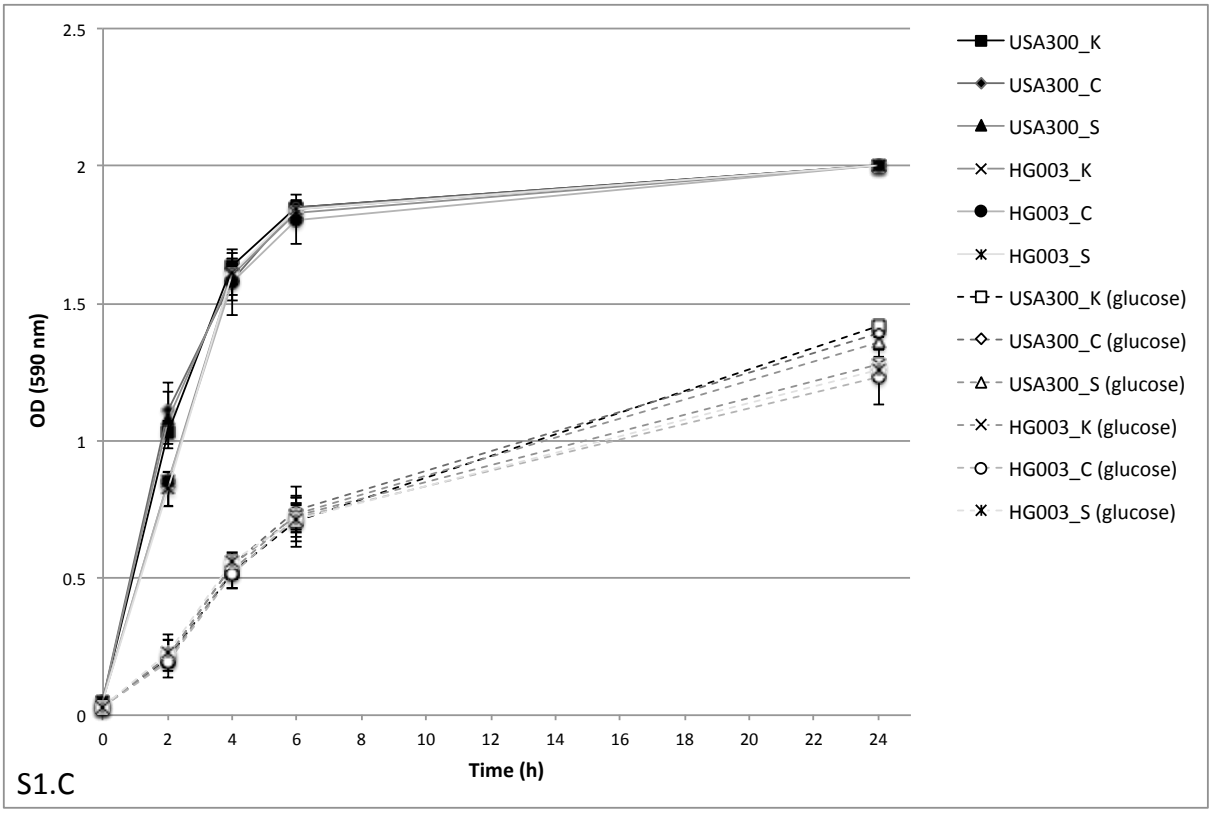
Time (h)
USA300_K
USA300_C
USA300_S
HG003_K
HG003_C
HG003_S
USA300_K (nitrite)
USA300_C (nitrite)
USA300_S (nitrite)
HG003_K (nitrite)
HG003_C (nitrite)
HG003_S (nitrite)

S1.B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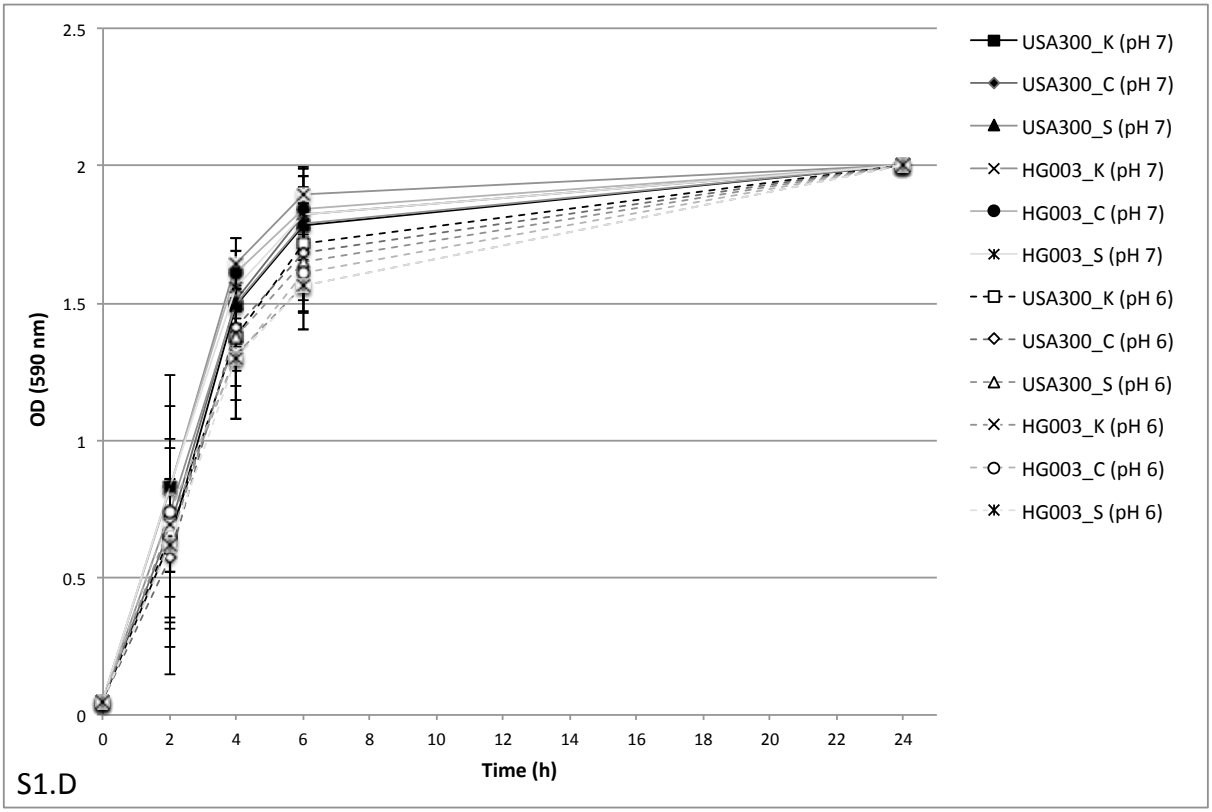
Time (h)
USA300_K
USA300_C
USA300_S
HG003_K
HG003_C
HG003_S
USA300_K (glucose)
USA300_C (glucose)
USA300_S (glucose)
HG003_K (glucose)
HG003_C (glucose)
HG003_S (glucose)

S1.C


OD (590 nm)
Time (h)
USA300_K (pH 7)
USA300_C (pH 7)
USA300_S (pH 7)
HG003_K (pH 7)
HG003_C (pH 7)
HG003_S (pH 7)
USA300_K (pH 6)
USA300_C (pH 6)
USA300_S (pH 6)
HG003_K (pH 6)
HG003_C (pH 6)
HG003_S (pH 6)

S1.D

Supplementary Table 1

seb promoter activity is presented using Vmax mean values generated in the β-lactamase assay. Promoter activity under control and stress conditions (4.5 % NaCl, 150 mg/L nitrite, 30% glucose, lactic acid pH 6.0) as well as in wild type strains and regulatory mutants (Δ*agr*, Δ*sigB*) was compared for each time point. Statistically significant changes are marked by an asterisk ($p < 0.05$).

Strain	Growth condition /regulatory mutation	Time (h)	Vmax (mean)	SD	p-value	*seb* promoter activity
USA300_K	NaCl	2	45.85	6.22	0.46	
	Nitrite		39.79	3.87	0.02*	↓
	Glucose		26.72	4.21	0.00*	↓
	Lactic acid		13.52	2.78	0.20	
	Δ*agr*		45.18	3.67	0.26	
	Δ*sigB*		56.40	8.21	0.16	
	NaCl	4	67.89	2.84	0.00*	↓
	Nitrite		171.63	56.76	0.03*	↓
	Glucose		33.99	7.82	0.00*	↓
	Lactic acid		202.14	54.03	0.01*	↑
	Δ*agr*		124.70	12.81	0.00*	↓
	Δ*sigB*		500.47	81.66	0.00*	↑
	NaCl	6	160.49	16.02	0.00*	↓
	Nitrite		349.96	69.17	0.19	
	Glucose		49.69	4.59	0.00*	↓
	Lactic acid		354.15	78.02	0.07	

	Δ*agr*		185.43	27.28	0.00*	↓
	Δ*sigB*		738.77	67.32	0.00*	↑
	NaCl	24	407.32	31.63	0.00*	↓
	Nitrite		567.61	38.79	0.02*	↑
	Glucose		57.57	4.80	0.00*	↓
	Lactic acid		503.43	19.64	0.00*	↑
	Δ*agr*		175.83	26.22	0.00*	↓
	Δ*sigB*		643.83	46.55	0.00*	↑
USA300_C	NaCl	2	37.86	6.36	0.01*	↓
	Nitrite		46.42	15.51	0.60	
	Glucose		26.17	4.04	0.00*	↓
	Lactic acid		13.66	4.45	0.69	
	Δ*agr*		44.49	3.06	0.10	
	Δ*sigB*		53.46	15.48	0.65	
	NaCl	4	66.90	5.67	0.00*	↓
	Nitrite		174.52	39.24	0.03*	↓
	Glucose		36.70	14.09	0.00*	↓
	Lactic acid		254.02	19.17	0.00*	↑
	Δ*agr*		161.62	18.47	0.01*	↓
	Δ*sigB*		527.55	19.02	0.00*	↑
	NaCl	6	147.82	8.85	0.00*	↓
	Nitrite		317.44	47.55	0.04*	↓
	Glucose		53.71	16.74	0.00*	↓
	Lactic acid		419.33	79.44	0.00*	↑
	Δ*agr*		262.00	21.45	0.00*	↓

	Δ*sigB*		739.33	7.35	0.00*	↑
	NaCl	24	401.29	23.74	0.05	
	Nitrite		563.36	18.01	0.45	
	Glucose		61.91	10.65	0.00*	↓
	Lactic acid		482.42	16.80	0.97	
	Δ*agr*		269.61	7.99	0.00*	↓
	Δ*sigB*		570.94	65.26	0.59	
USA300_S	NaCl	2	34.49	8.21	0.02*	↓
	Nitrite		39.45	3.22	0.02*	↓
	Glucose		22.58	3.92	0.00*	↓
	Lactic acid		19.08	8.33	0.30	
	Δ*agr*		30.79	4.05	0.00*	↓
	Δ*sigB*		54.89	8.23	0.07	
	NaCl	4	58.56	2.87	0.00*	↓
	Nitrite		158.58	47.26	0.00*	↓
	Glucose		31.05	9.92	0.00*	↓
	Lactic acid		224.25	7.62	0.00*	↑
	Δ*agr*		136.00	23.07	0.00*	↓
	Δ*sigB*		488.61	65.99	0.00*	↑
	NaCl	6	126.08	11.84	0.00*	↓
	Nitrite		327.23	40.58	0.01*	↓
	Glucose		53.28	22.05	0.00*	↓
	Lactic acid		427.59	83.70	0.11	
	Δ*agr*		219.74	35.08	0.01*	↓
	Δ*sigB*		768.27	70.40	0.00*	↑

	NaCl	24	470.88	38.16	0.00*	↓
	Nitrite		568.39	24.91	0.15	
	Glucose		62.73	10.34	0.00*	↓
	Lactic acid		526.29	18.15	0.00*	↑
	Δ*agr*		222.10	16.67	0.00*	↓
	Δ*sigB*		669.38	19.20	0.00*	↑
HG003_K	NaCl	2	24.77	9.29	0.99	
	Nitrite		25.50	3.36	0.75	
	Glucose		26.78	6.86	0.59	
	Lactic acid		14.78	2.71	0.04*	↑
	Δ*agr*		21.20	8.53	0.43	
	Δ*sigB*		21.81	2.57	0.24	
	NaCl	4	29.60	3.34	0.00*	↓
	Nitrite		36.00	1.56	0.00*	↓
	Glucose		31.30	5.40	0.00*	↓
	Lactic acid		51.28	15.44	0.23	
	Δ*agr*		31.57	3.87	0.00*	↓
	Δ*sigB*		121.25	13.69	0.00*	↑
	NaCl	6	38.56	5.98	0.00*	↓
	Nitrite		55.13	4.83	0.00*	↓
	Glucose		26.94	4.57	0.00*	↓
	Lactic acid		66.98	6.84	0.03*	↓
	Δ*agr*		67.00	9.66	0.02*	↓
	Δ*sigB*		438.71	23.06	0.00*	↑
	NaCl	24	76.64	12.48	0.00*	↓

	Nitrite		79.13	20.68	0.01*	↓
	Glucose		25.94	3.72	0.00*	↓
	Lactic acid		78.39	2.91	0.00*	↑
	Δ*agr*		66.04	15.36	0.00*	↓
	Δ*sigB*		402.52	12.09	0.00*	↑
HG003_C	NaCl	2	24.33	4.45	0.20	
	Nitrite		28.61	12.01	0.17	
	Glucose		23.66	2.19	0.12	
	Lactic acid		17.10	5.70	0.32	
	Δ*agr*		24.56	22.41	0.72	
	Δ*sigB*		17.38	3.63	0.07	
	NaCl	4	28.31	4.38	0.00*	↓
	Nitrite		42.10	6.10	0.30	
	Glucose		37.75	21.93	0.40	
	Lactic acid		65.84	17.04	0.82	
	Δ*agr*		29.86	3.17	0.00*	↓
	Δ*sigB*		105.44	29.82	0.00*	↑
	NaCl	6	41.26	5.51	0.00*	↓
	Nitrite		59.12	6.96	0.00*	↓
	Glucose		22.81	5.13	0.00*	↓
	Lactic acid		80.90	25.37	0.85	
	Δ*agr*		59.18	4.55	0.00*	↓
	Δ*sigB*		379.90	89.01	0.00*	↑
	NaCl	24	65.64	10.42	0.00*	↓
	Nitrite		78.14	30.45	0.24	

	Glucose		26.91	4.82	0.00*	↓
	Lactic acid		98.46	33.05	0.25	
	Δ*agr*		64.34	13.54	0.00*	↓
	Δ*sigB*		431.77	21.27	0.00*	↑
HG003_S	NaCl	2	14.46	2.48	0.05	
	Nitrite		15.39	1.40	0.08	
	Glucose		14.65	1.85	0.59	
	Lactic acid		12.99	3.79	0.57	
	Δ*agr*		14.66	0.97	0.05	
	Δ*sigB*		35.84	6.19	0.01*	↑
	NaCl	4	16.93	3.92	0.10	
	Nitrite		18.08	4.92	0.23	
	Glucose		15.28	3.74	0.04*	↓
	Lactic acid		28.47	13.82	0.08	
	Δ*agr*		15.95	2.65	0.03*	↓
	Δ*sigB*		156.08	15.66	0.00*	↑
	NaCl	6	17.79	4.90	0.00*	↓
	Nitrite		22.21	2.21	0.00*	↓
	Glucose		13.99	6.14	0.00*	↓
	Lactic acid		30.38	6.03	0.00*	↓
	Δ*agr*		34.13	1.29	0.00*	↓
	Δ*sigB*		670.35	37.40	0.00*	↑
	NaCl	24	35.08	11.98	0.00*	↓
	Nitrite		75.31	18.38	0.00*	↓
	Glucose		19.52	5.19	0.00*	↓

Lactic acid	57.25	4.01	0.25	
Δ*agr*	62.42	6.93	0.00*	↓
Δ*sigB*	484.96	16.68	0.00*	↑

Highlights

- NaCl, nitrite, and glucose stress decrease *seb* promoter activity
- Lactic acid stress (pH 6.0) leads to increased *seb* promoter activity
- Loss of *agr* decreases and loss of *sigB* increases *seb* promoter activity
- *seb* promoter activity is not dependent on 1 kb sequence upstream of *seb*